

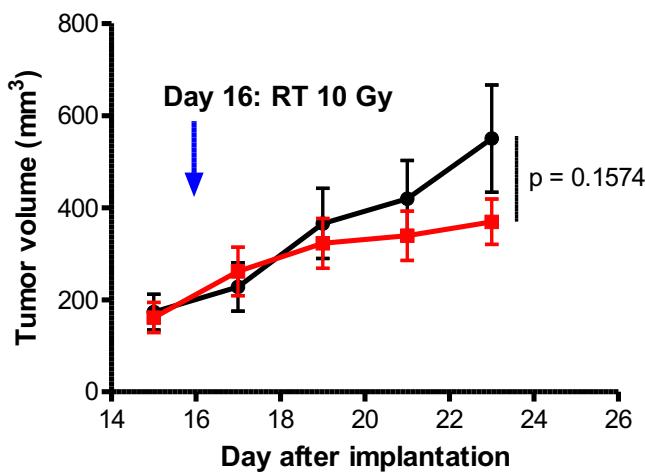
# Supplementary Data

**Supplementary Figure 1**

**RENCA**

**A**

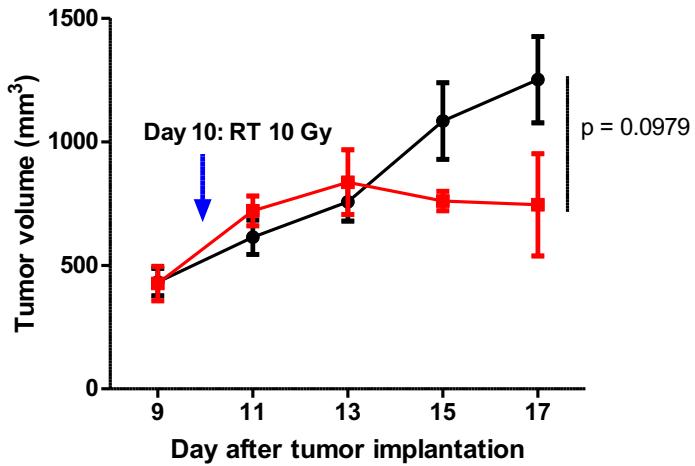
● Control  
■ RT



**MC38**

**B**

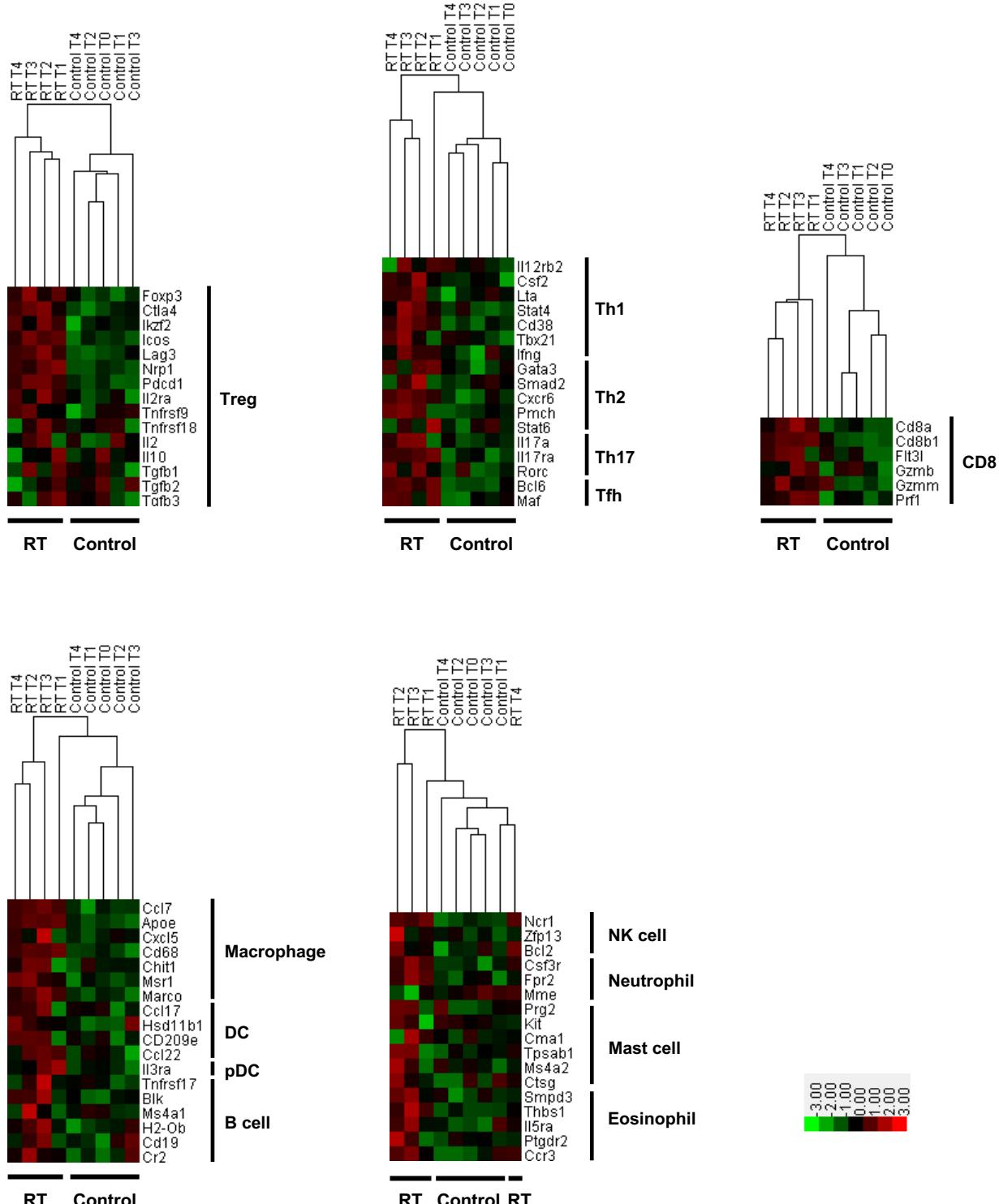
● Control  
■ RT



**Supplementary Figure 1: Radiotherapy suppresses tumor growth.**

(A, B) Tumor volume curves of RENCA (A) and MC38 (B) bearing mice. Non-irradiated tumors in black line and irradiated tumors in red line, respectively. N = 5-15 per group, repeated at least 4x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired t-test at the day of harvest (A, B).

## Supplementary Figure 2

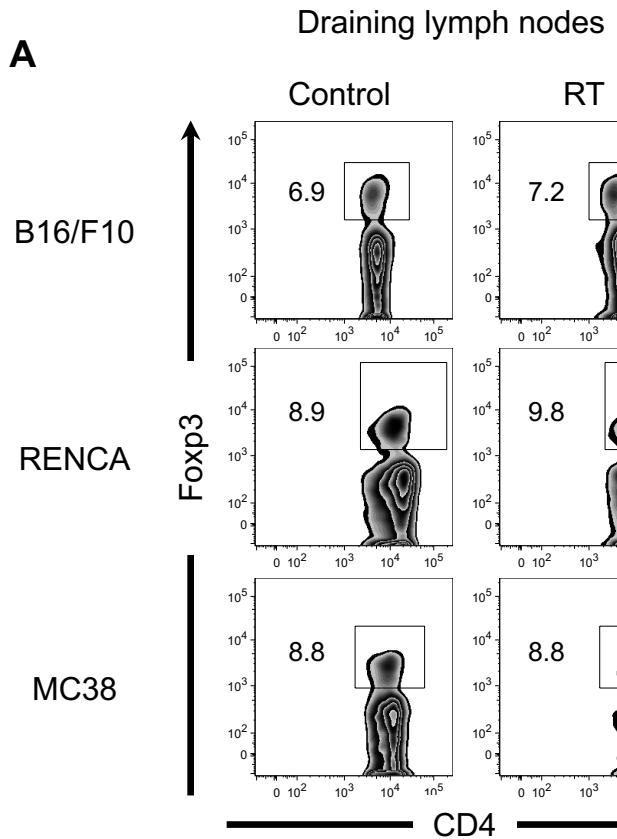


**Supplementary Figure 2: Characterization of the TME of B16/F10 tumors post-RT.**

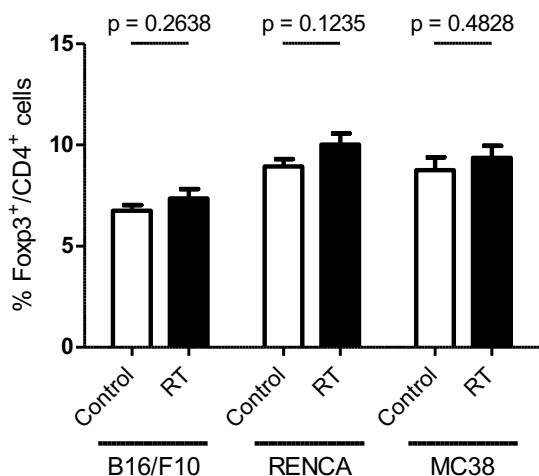
Heatmaps of RNA expression of genes associated with immune cell subsets by NanoString assay. RNAs were extracted from the tumors on day 14 B16/F10 tumor-bearing mice (7 days post RT). N= 4-5 per group.

### Supplementary Figure 3

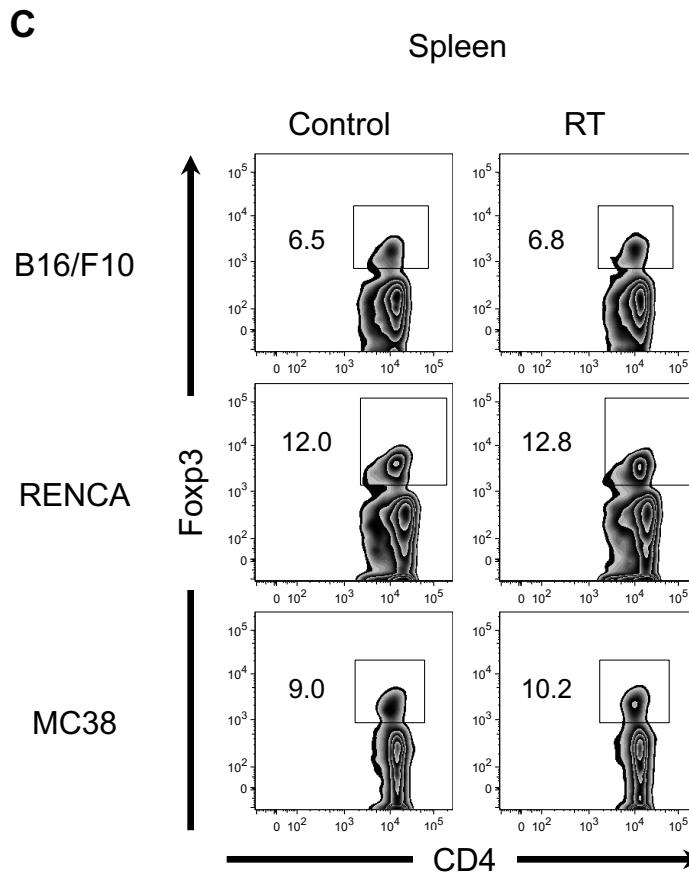
**A**



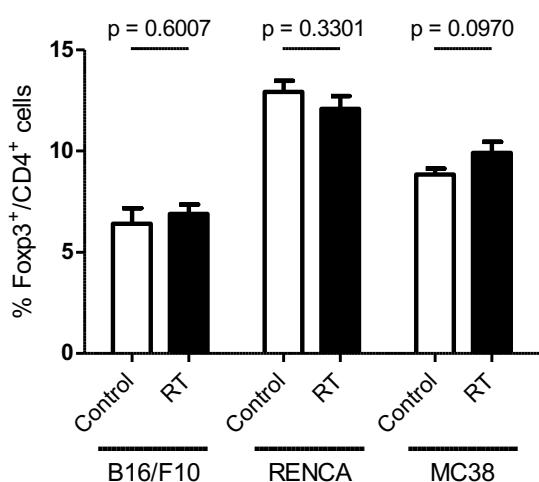
**B**



**C**



**D**

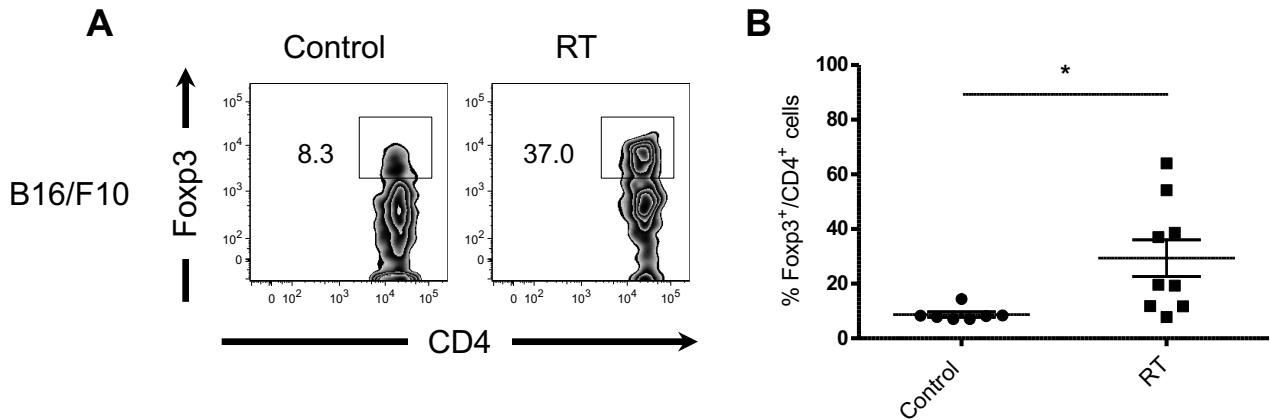


**Supplementary Figure 3: Stereotactic radiation does not change Treg in draining lymph nodes nor in spleens of treated mice.**

(A, C) Representative flow plots of Treg from DLN (A) and spleen (C) from B16/F10, RENCA, or MC38 bearing mice. (B, D) Bar graph of % Foxp3<sup>+</sup> cells in CD4<sup>+</sup> cells from DLN (B) and spleen (D). N = 7 - 14 per group, repeated at least 4x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05.

## Supplementary Figure 4

Day 21 (14 days post-RT) B16/F10 TIL

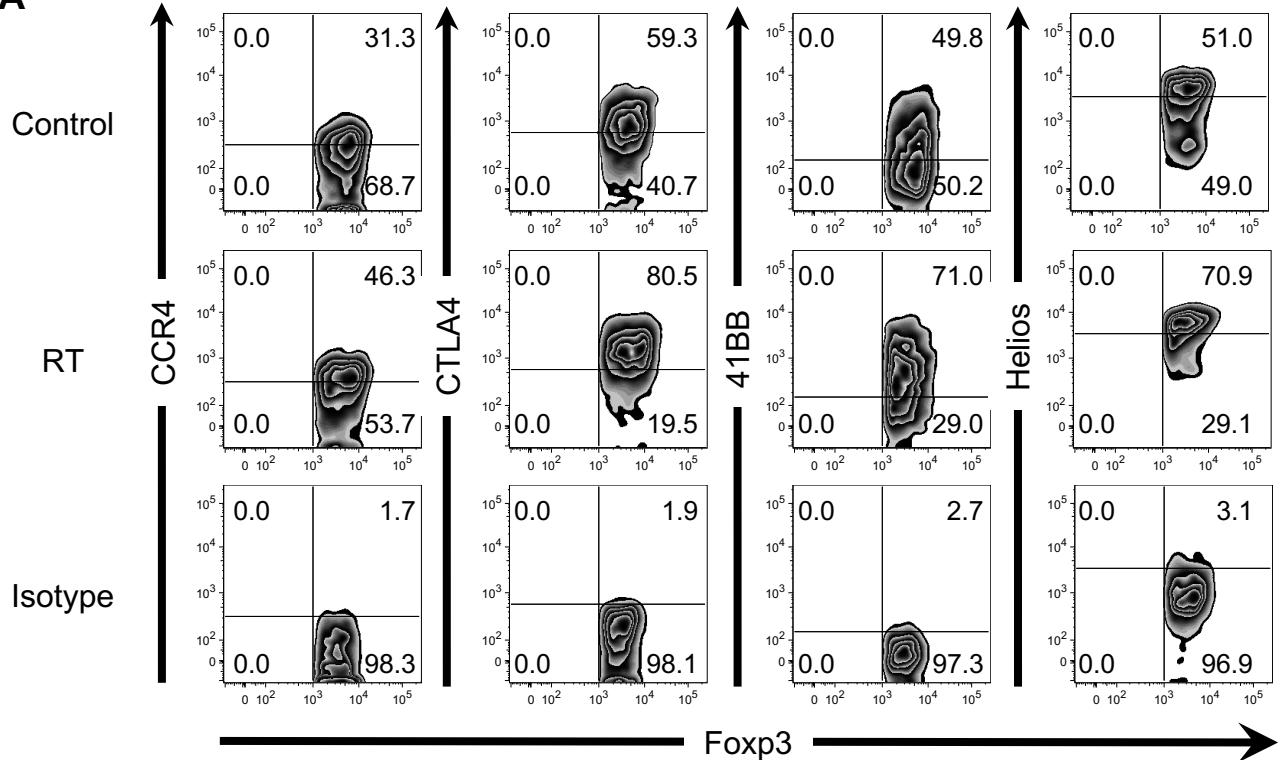


**Supplementary Figure 4: Persistently Increased Treg Post-RT.**

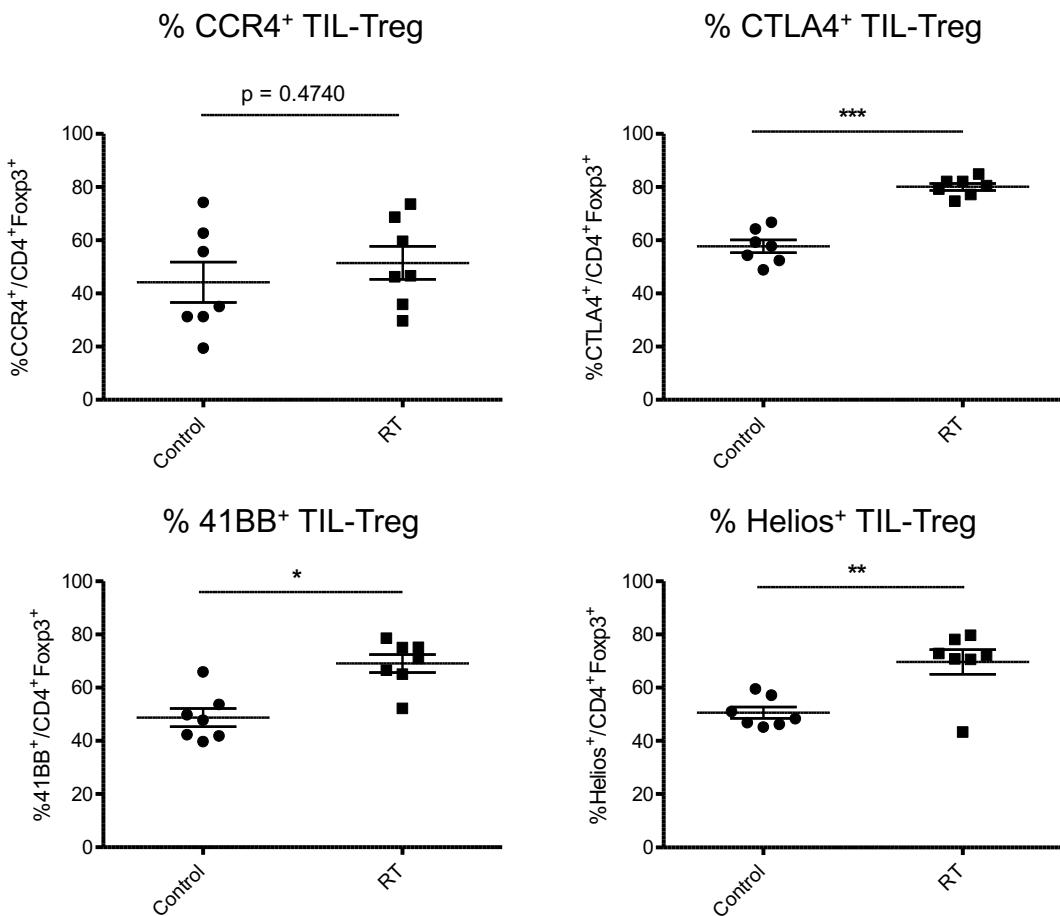
Representative flow plots (A) and quantitative scatterplot (B) depicting of the percentages of  $\text{Foxp3}^+$  cells in  $\text{CD4}^+$  cells from TILs, from day 21 (14 days post-RT) B16/F10 tumor-bearing mice. N= 7-9 per group, repeated 2x. Error bars represent SEM, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 5

**A**



**B**



**Supplementary Figure 5: Stereotactic radiation increases the suppressive markers of Treg in the RENCA tumor model.**

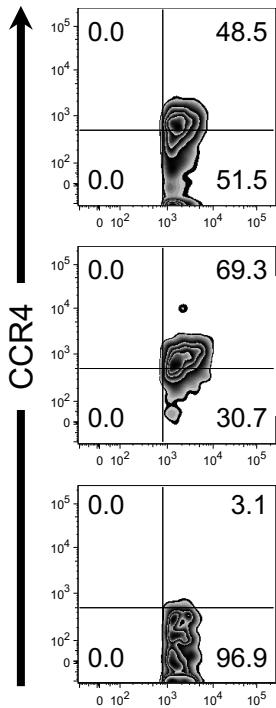
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of CCR4<sup>+</sup>, CTLA-4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> TIL-Treg, from day 23 RENCA-bearing mice. N = 7 per group, repeated 3x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 6

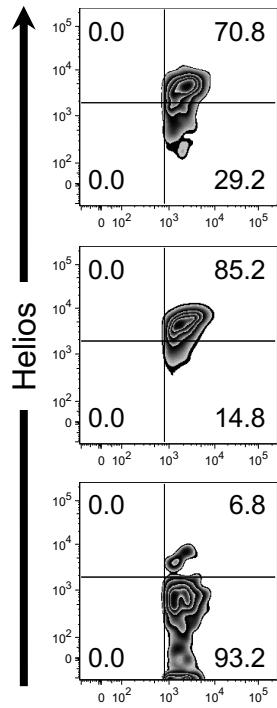
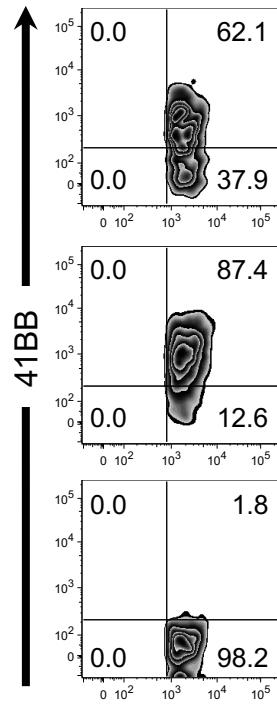
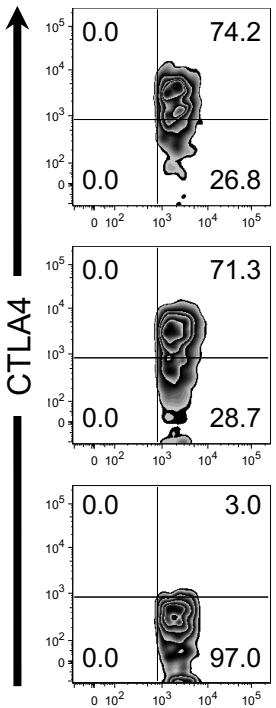
**A**

MC38 TIL-Treg

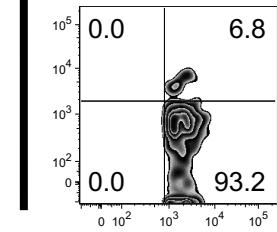
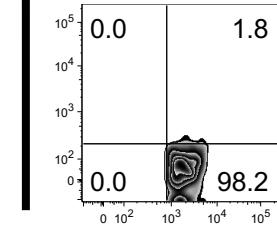
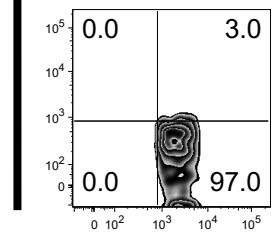
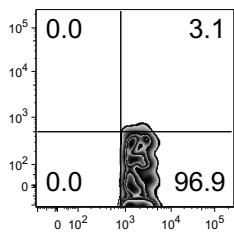
Control



RT



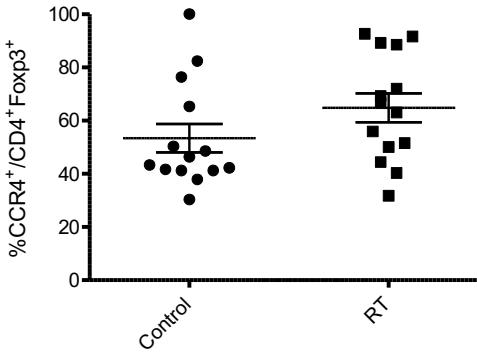
Isotype



Foxp3 →

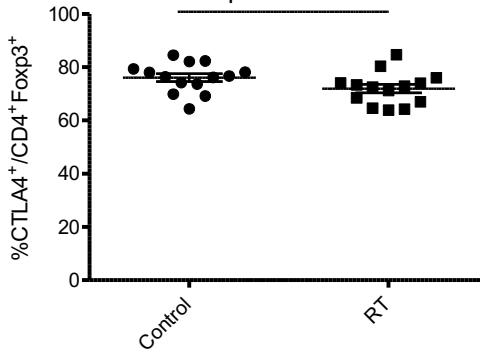
% CCR4<sup>+</sup> TIL-Treg

$p = 0.1453$



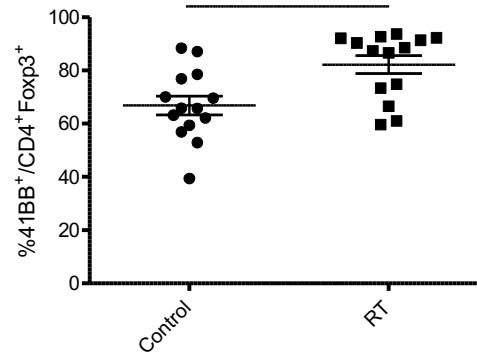
% CTLA4<sup>+</sup> TIL-Treg

$p = 0.0717$



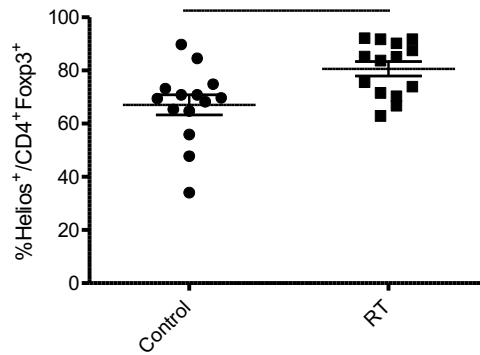
% 41BB<sup>+</sup> TIL-Treg

\*\*



% Helios<sup>+</sup> TIL-Treg

\*\*



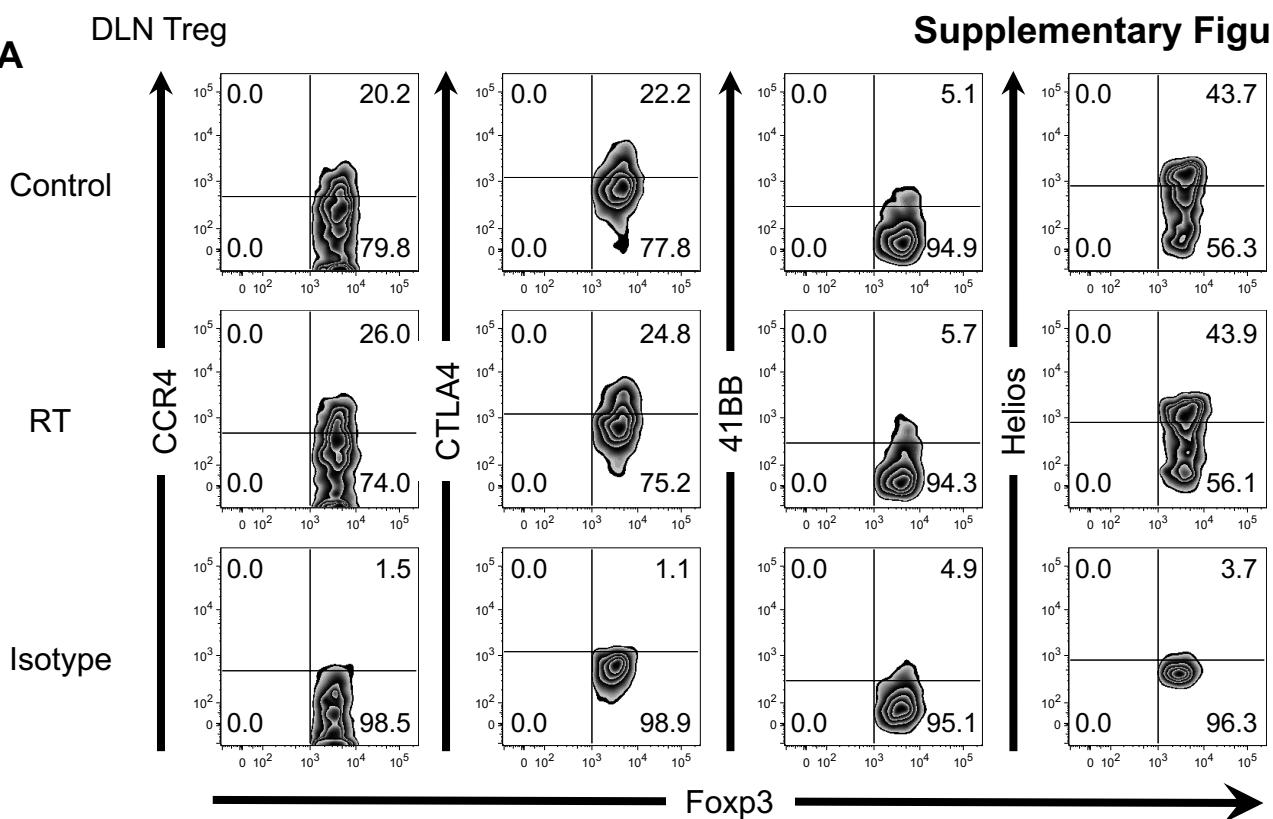
**B**

**Supplementary Figure 6: Stereotactic radiation increases the suppressive markers of Treg in the MC38 tumor model.**

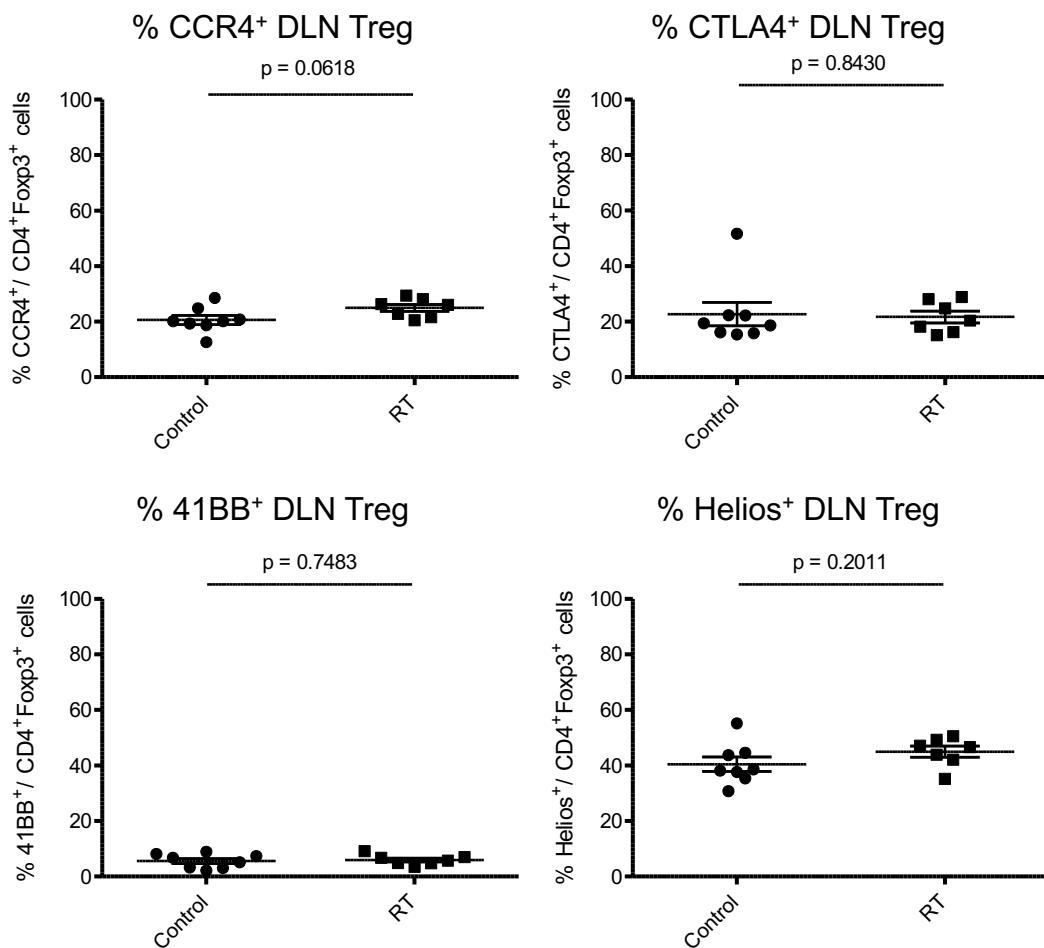
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of CCR4<sup>+</sup>, CTLA4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> TIL-Treg, from day 17 MC38-bearing mice. N = 14 per group, repeated x2. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 7

**A**



**B**

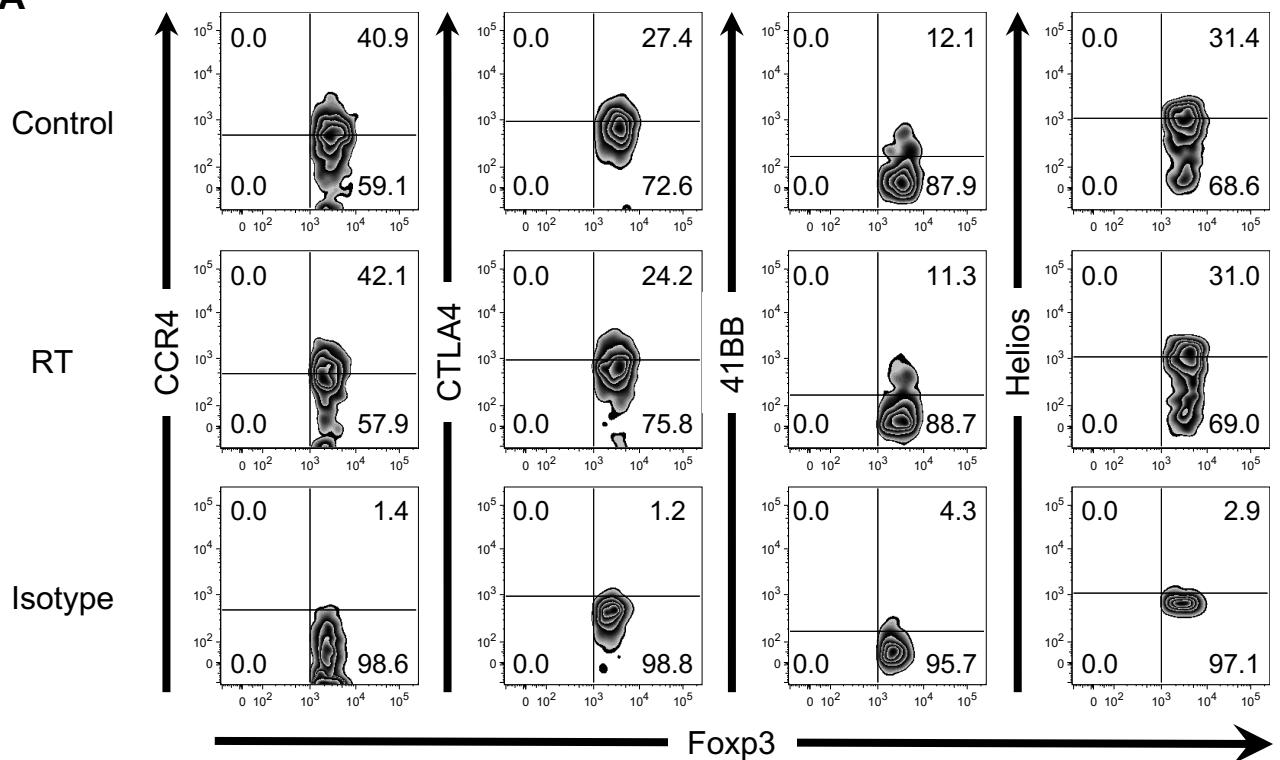


**Supplementary Figure 7: Stereotactic radiation does not change the expression of the suppressive markers of Treg in DLNs.**

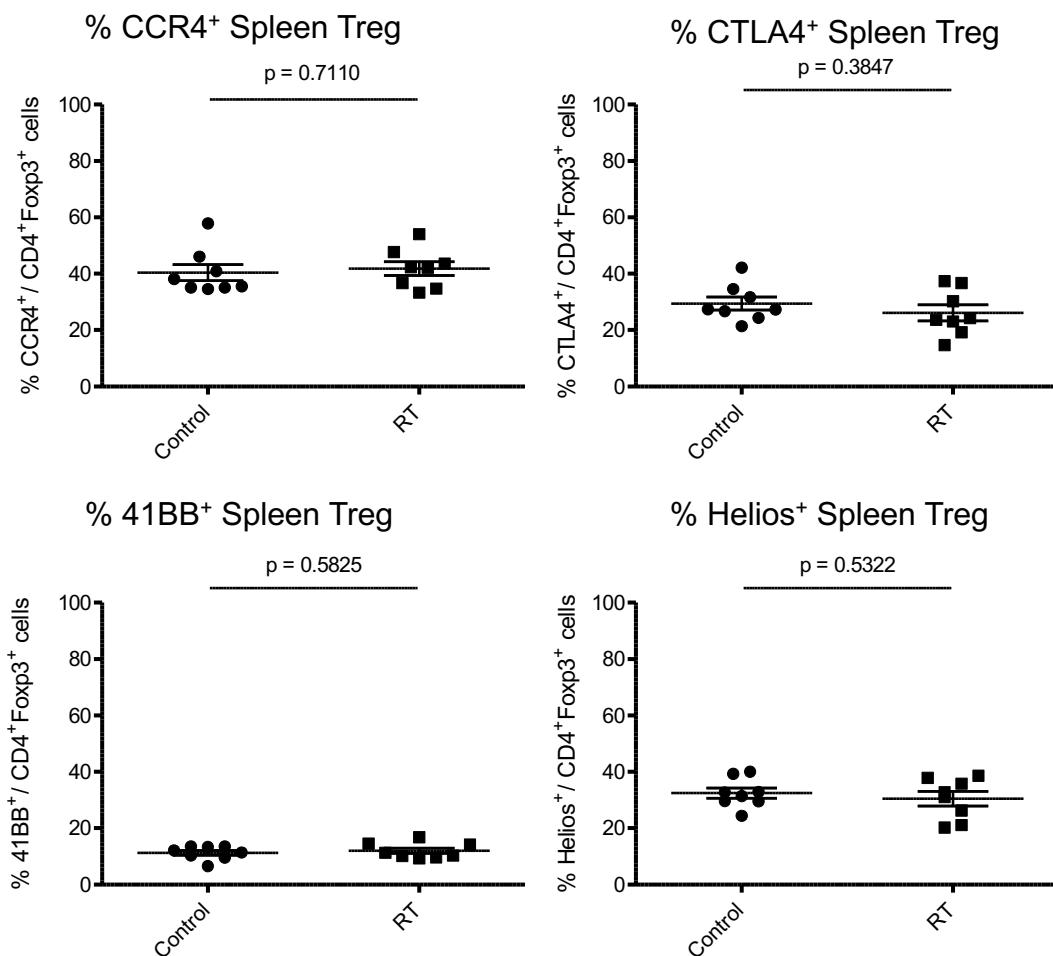
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of CCR4<sup>+</sup>, CTLA4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> Treg, from DLNs of day 14 B16/F10-bearing mice. N = 8 per group, repeated 3x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 8

## A Spleen Treg



## B

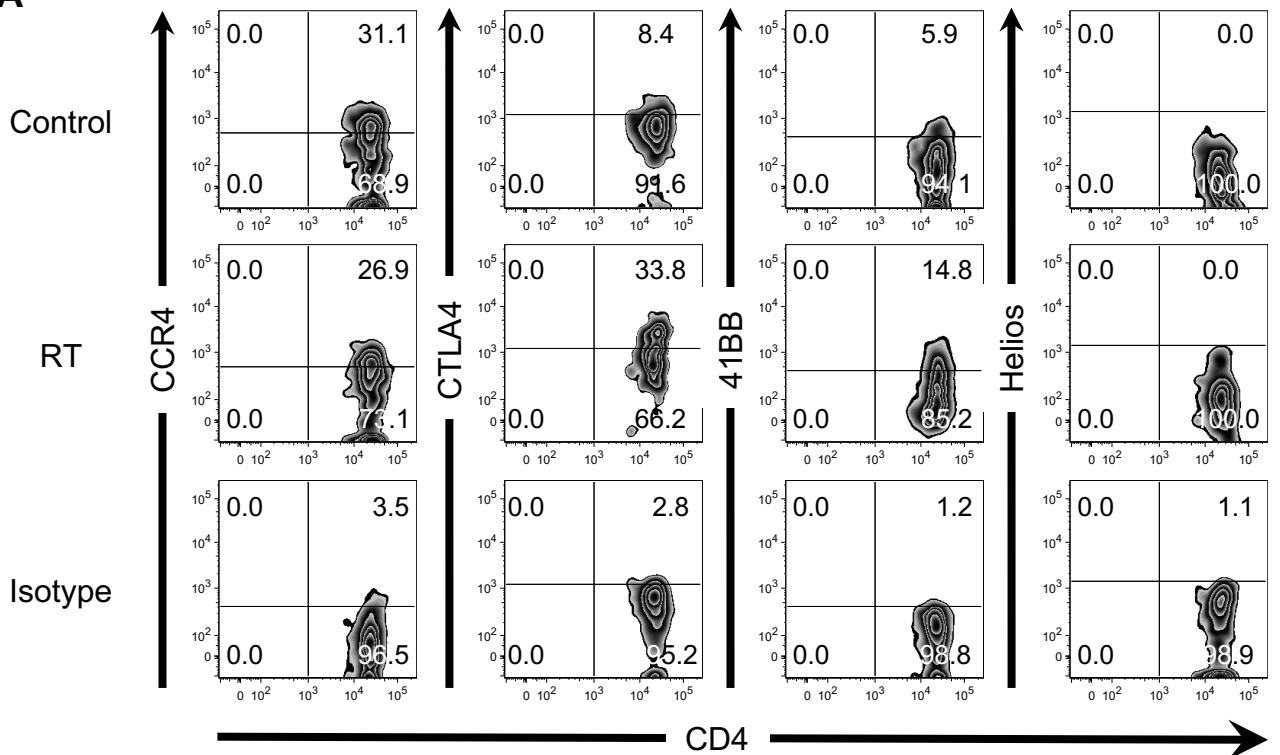


**Supplementary Figure 8: Stereotactic radiation does not change the expression of the suppressive markers of Treg in spleens.**

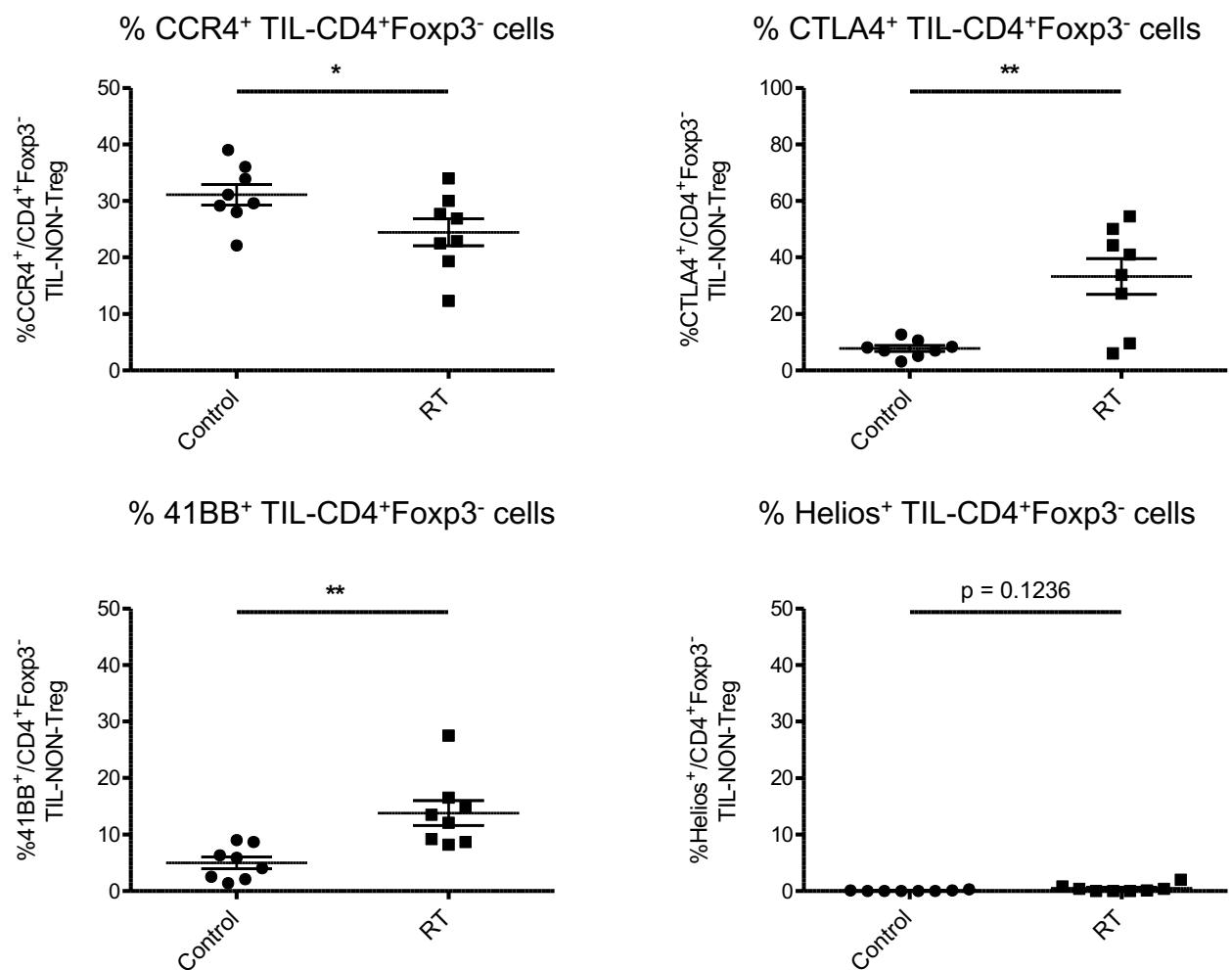
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting of the percentages of CCR4<sup>+</sup>, CTLA4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> Treg, from spleens of day 14 B16/F10-bearing mice. N = 8 per group, repeated 3x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 9

**A**



**B**

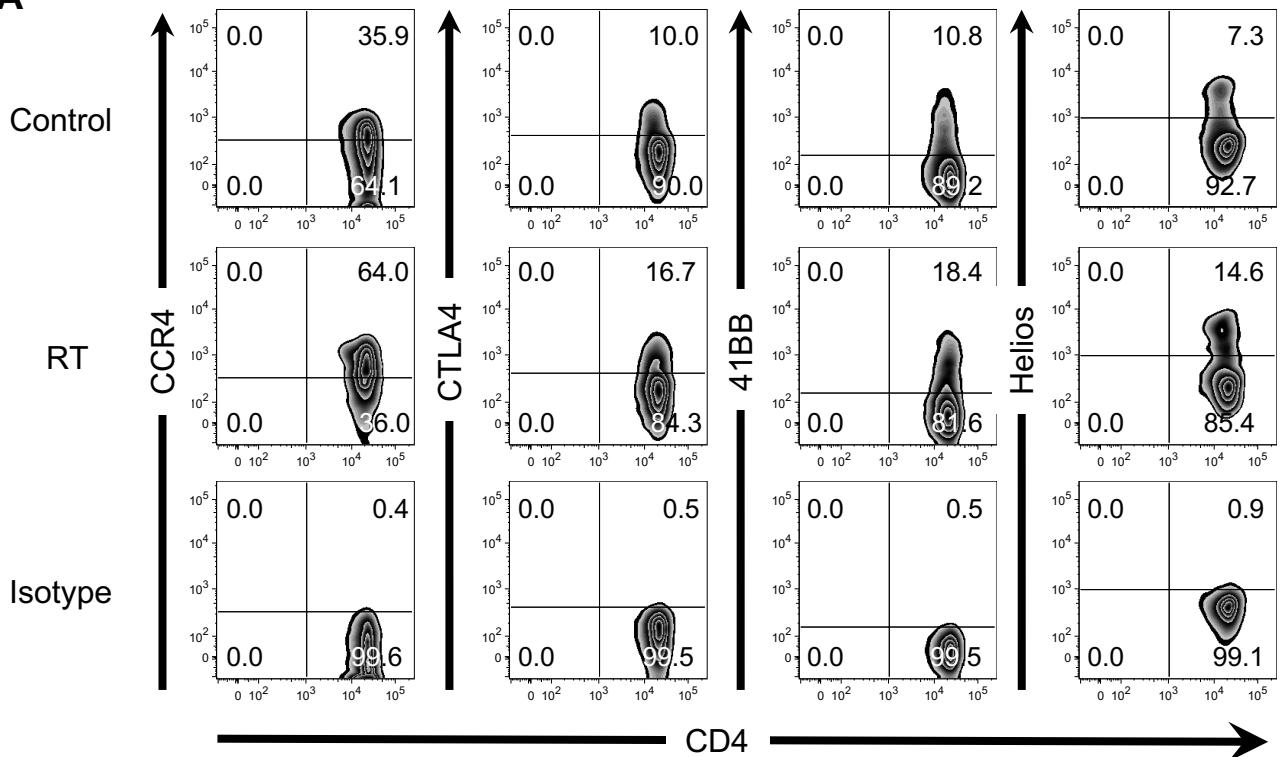


**Supplementary Figure 9: Expression of the selected markers of TIL-CD4<sup>+</sup>Foxp3<sup>-</sup> cells (Tconv) in the B16/F10 model.**

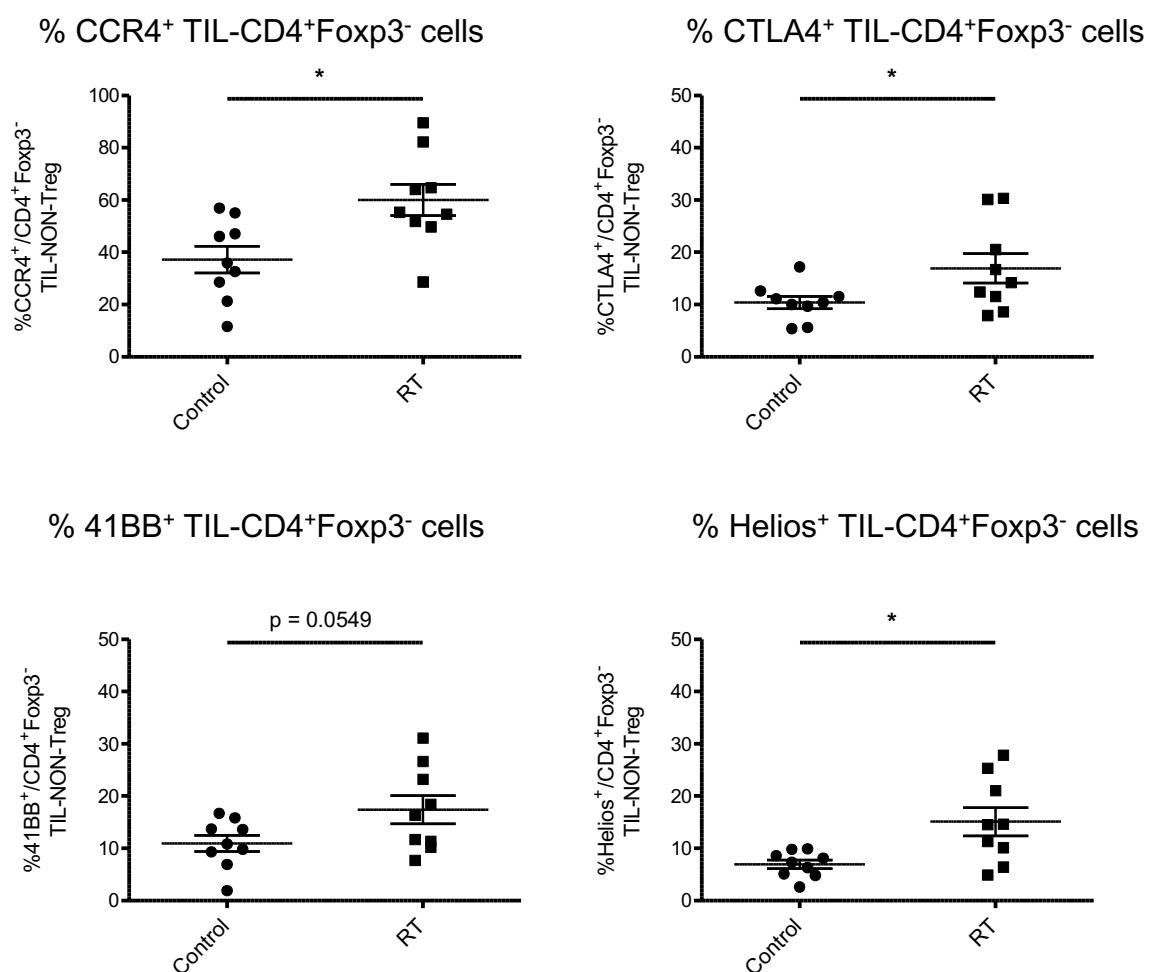
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of CCR4<sup>+</sup>, CTLA-4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> TIL-CD4<sup>+</sup>Foxp3<sup>-</sup> cells, from day 14 B16/F10-bearing mice. N = 8 per group, repeated 3x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 10

**A**



**B**

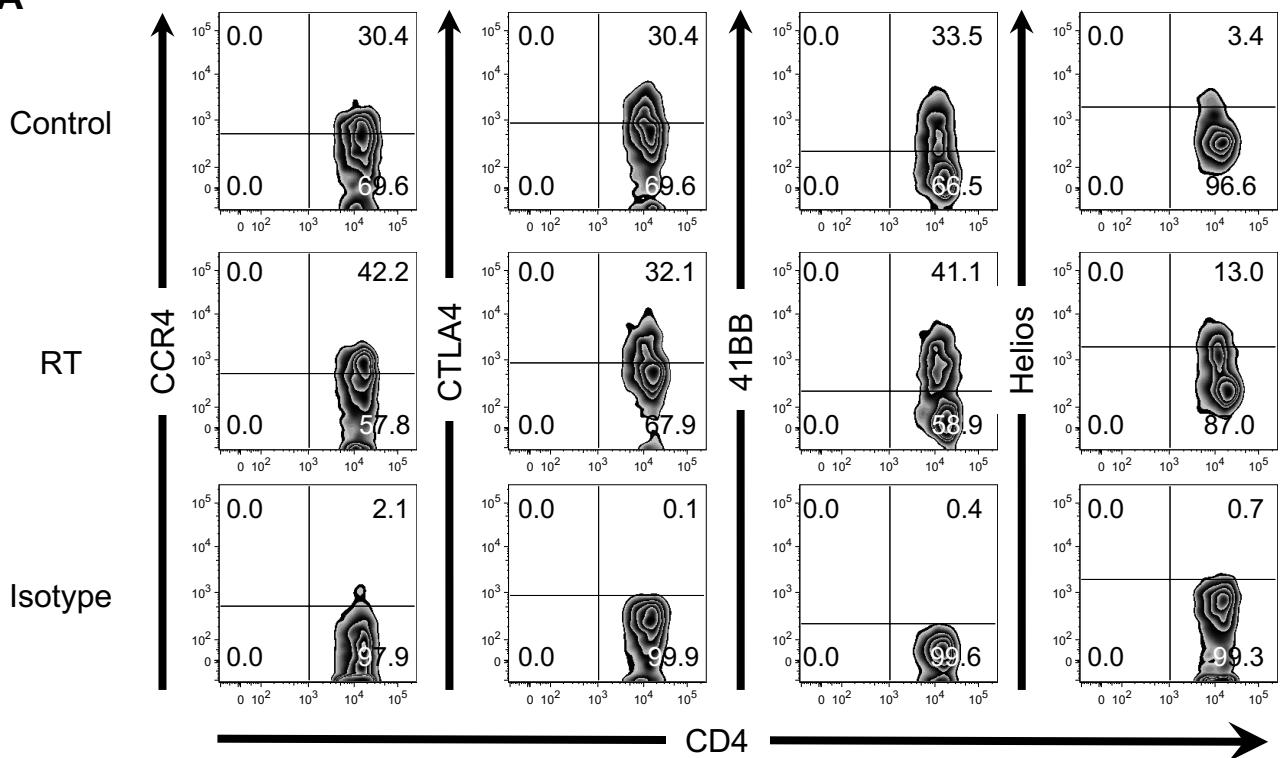


**Supplementary Figure 10: Expression of the selected markers of TIL-CD4<sup>+</sup>Foxp3<sup>-</sup> cells (Tconv) in the RENCA model.**

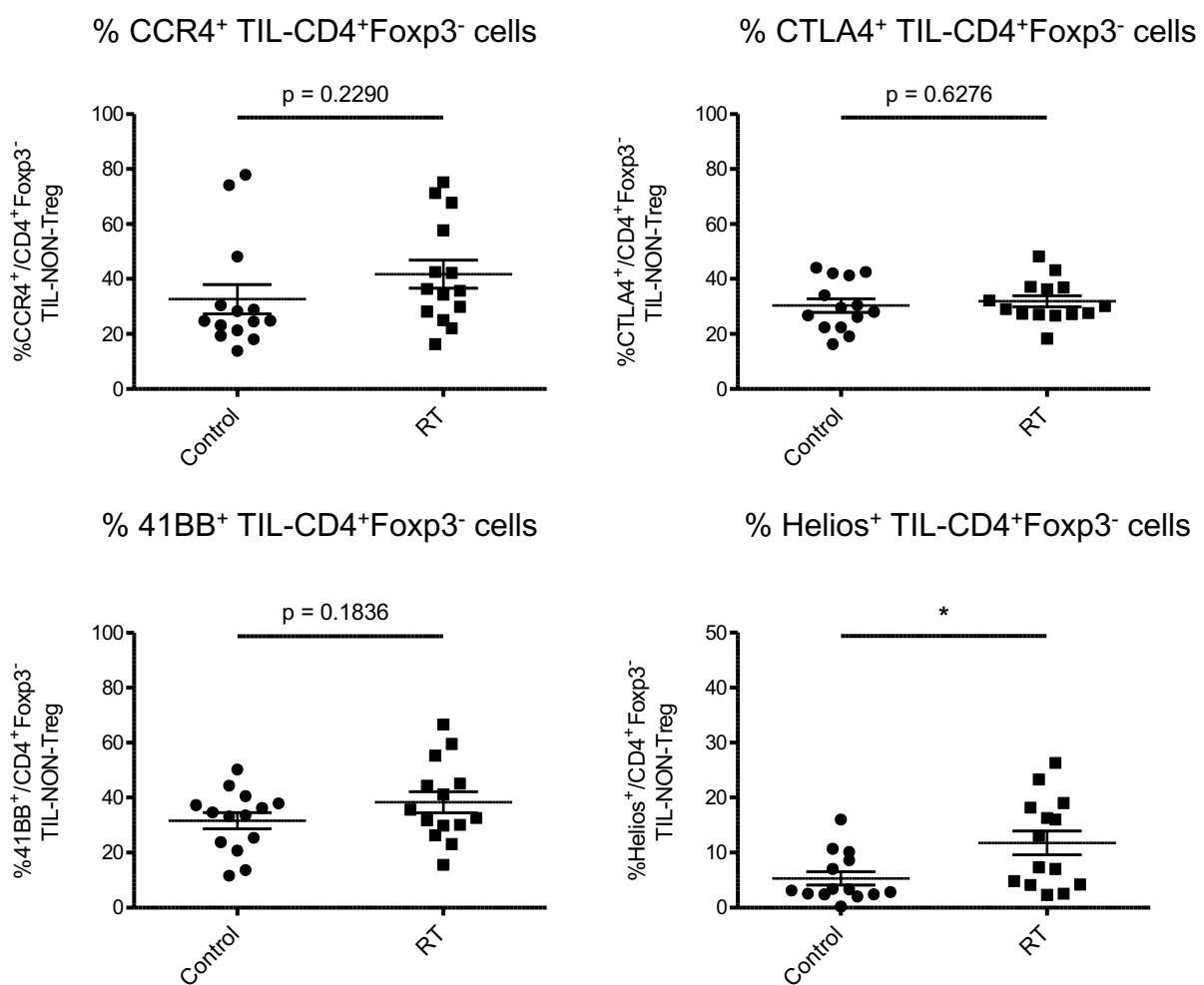
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of CCR4<sup>+</sup>, CTLA-4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> TIL-CD4<sup>+</sup>Foxp3<sup>-</sup> cells, from day 23 RENCA-bearing mice. N = 9 per group, repeated 3x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

# Supplementary Figure 11

**A**



**B**

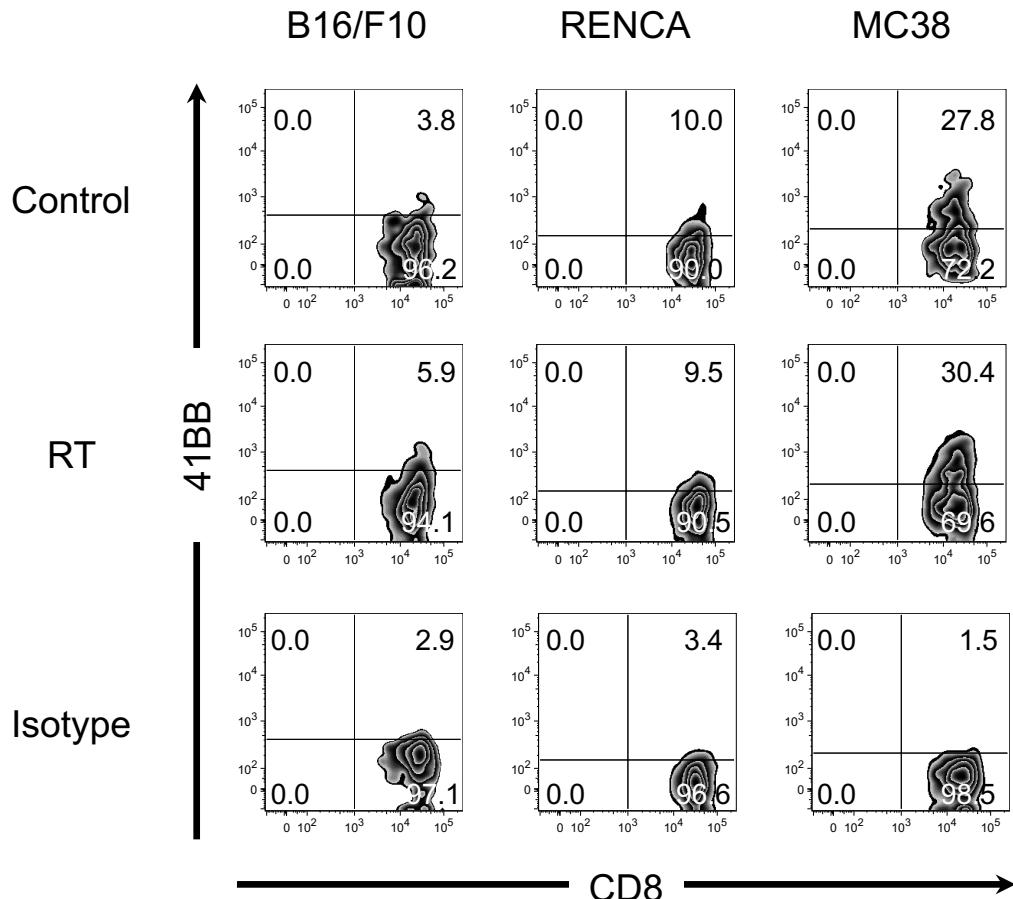


**Supplementary Figure 11: Expression of the selected markers of TIL-CD4<sup>+</sup>Foxp3<sup>-</sup> cells (Tconv) in the MC38 model.**

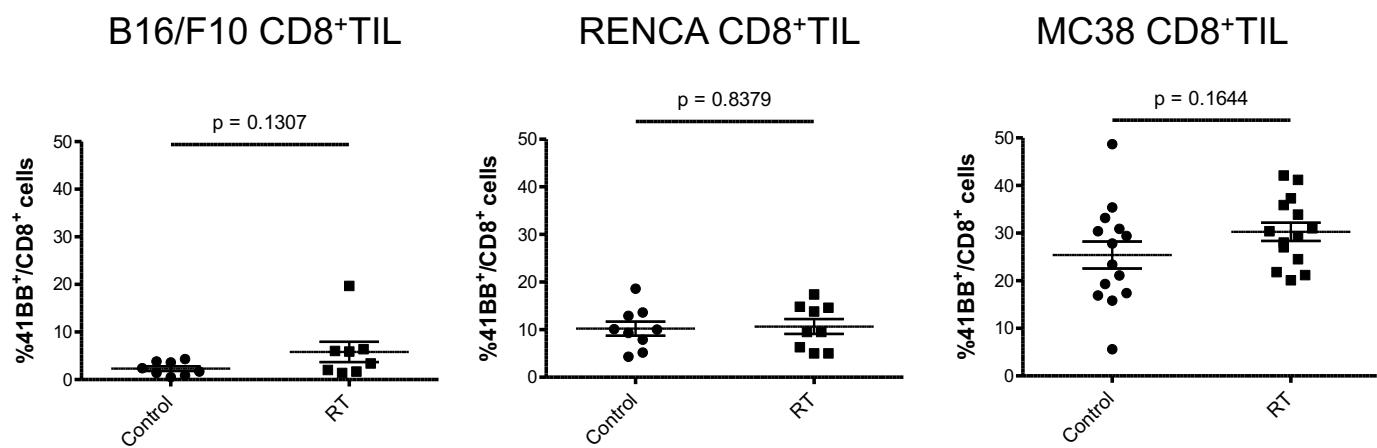
(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of CCR4<sup>+</sup>, CTLA-4<sup>+</sup>, 4-1BB<sup>+</sup> or Helios<sup>+</sup> TIL-CD4<sup>+</sup>Foxp3<sup>-</sup> cells, from day 17 MC38-bearing mice. N = 14 per group, repeated 2x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

## Supplementary Figure 12

**A**

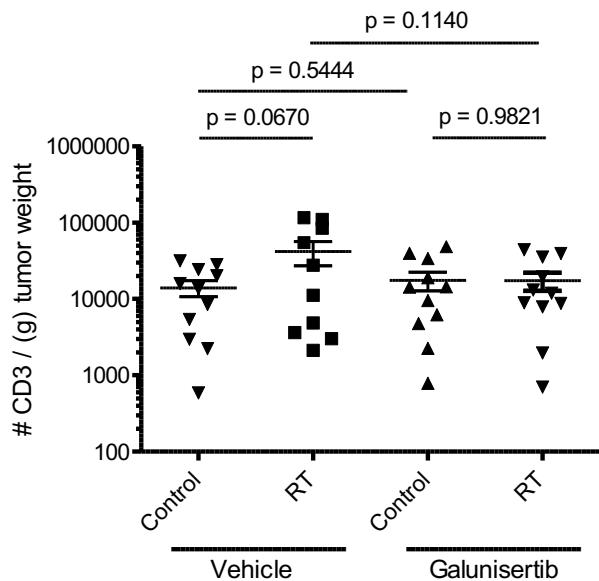
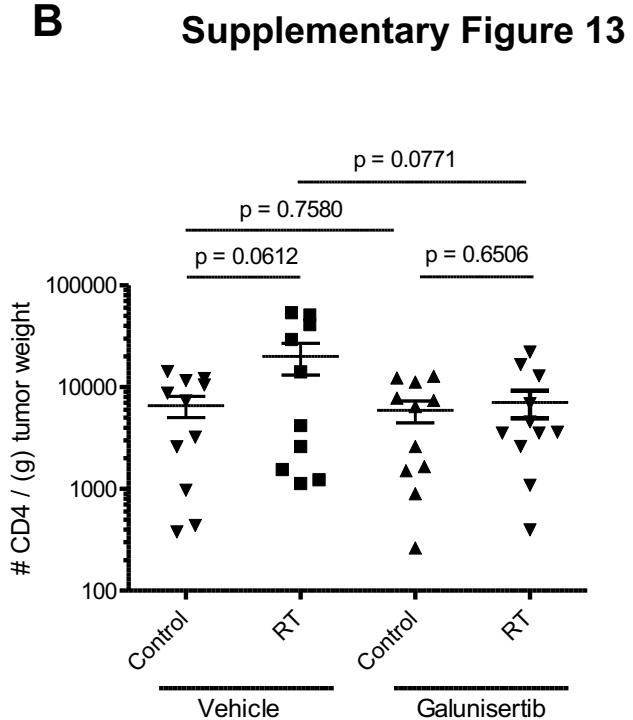
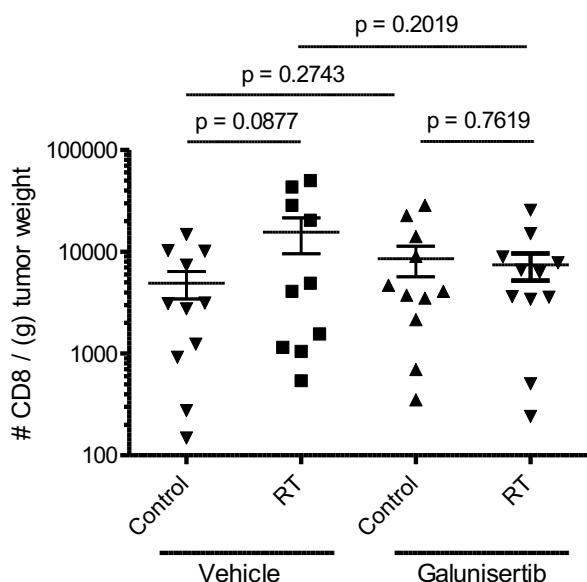


**B**



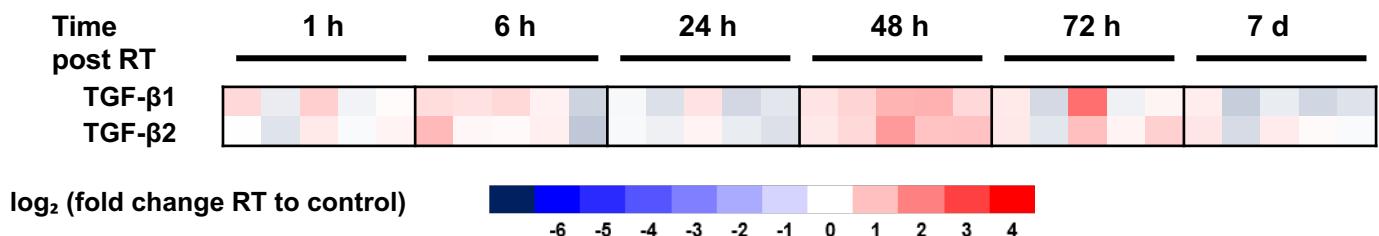
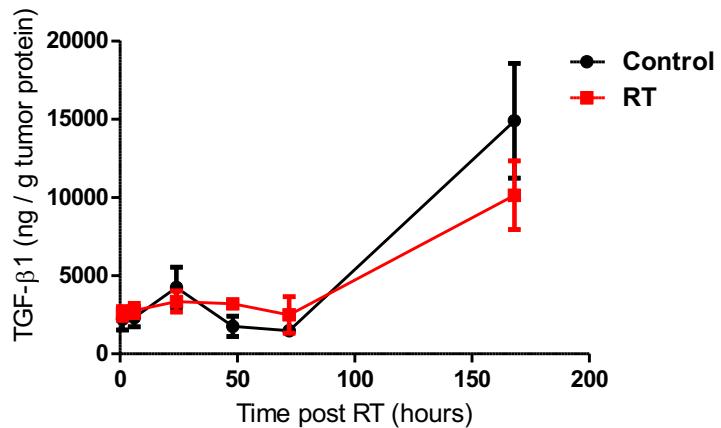
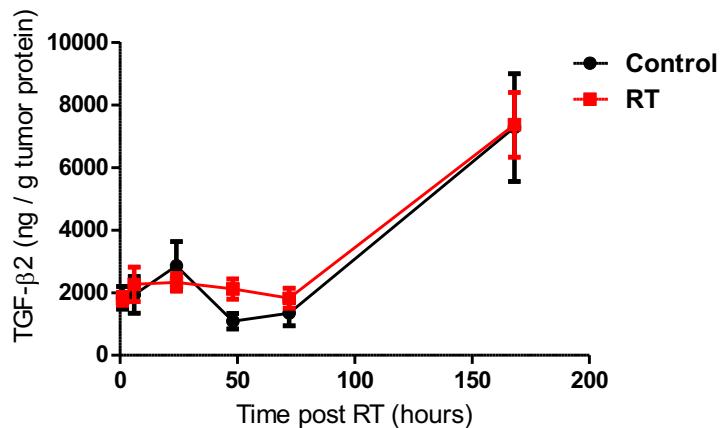
**Supplementary Figure 12: Expression of 4-1BB on TIL-CD8<sup>+</sup> cells.**

(A, B) Representative flow plots (A) and quantitative scatterplot (B) depicting the percentages of 4-1BB<sup>+</sup>TIL-CD8<sup>+</sup> cells, from day 14 B16/F10-bearing mice, day 23 RENCA-bearing mice and day 17 MC38-bearing mice. N = 8-14 per group, repeated 2-3x. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (B).

**A****B****C**

**Supplementary Figure 13: The effect of TGF- $\beta$  blockade on different T cell subsets.**

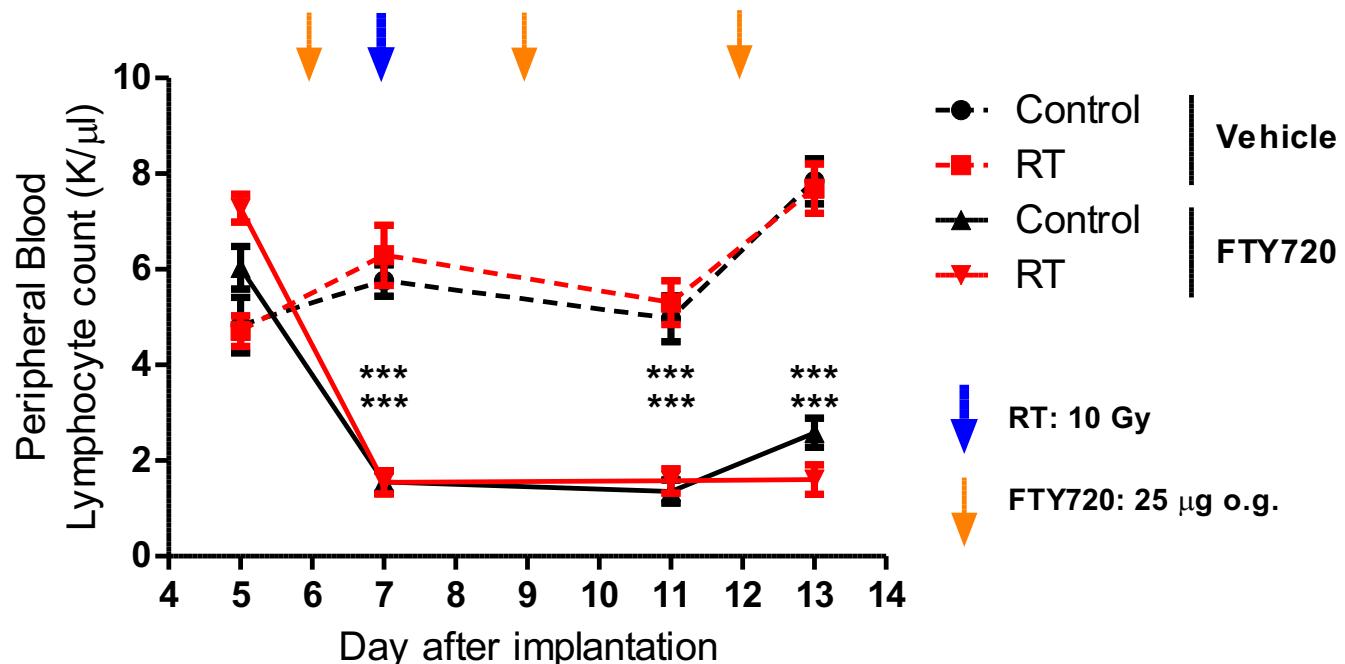
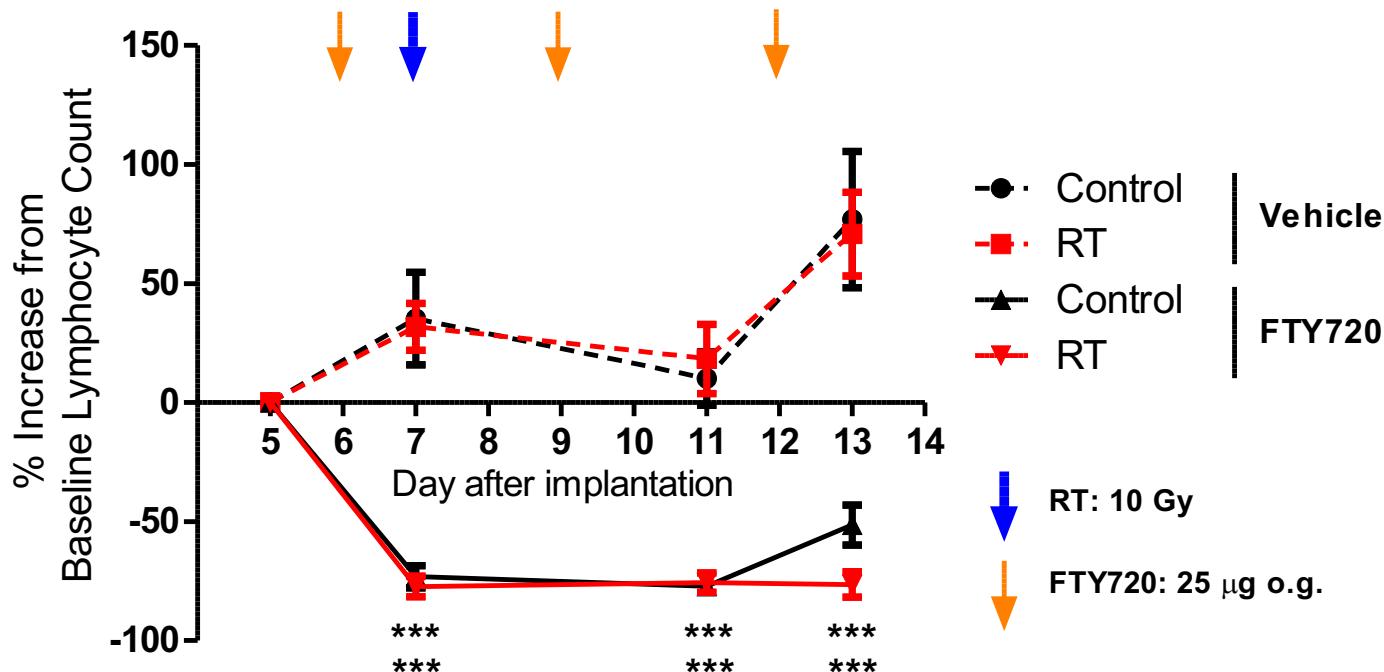
(A-C) The absolute numbers of tumor-infiltrating CD3 $^{+}$  (A), CD4 $^{+}$  (B) and CD8 $^{+}$  (C) cells per gram tumor weight from vehicle treated and Galunisertib treated groups, with or without radiation, from day 14 B16/F10-bearing mice are shown. N = 5 per group, repeated x2. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined by unpaired Student's t-test (A-C).

**A****Supplementary Figure 14****B****C**

**Supplementary Figure 14: TGF- $\beta$  expression in the tumor microenvironment.**

(A-C) Female Balb/c mice were subcutaneously implanted with  $3 \times 10^5$  RENCA cells and received 10 Gy RT as previously described. Tumor lysates were collected at different time points post RT and the intratumoral levels of TGF- $\beta$ 1 and TGF- $\beta$ 2 were analyzed by Luminex assay. N = 4 - 5 per group. (A) Heatmap of  $\log_2$  (fold change of radiated tumor chemokine levels compared to those of non-radiated control tumors). (B, C) Time courses of TGF- $\beta$ 1 and TGF- $\beta$ 2. TGF- $\beta$  levels were normalized to tumor protein concentration. N = 4 - 5 per group. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined two-way ANOVA (B, C).

Supplementary Figure 15

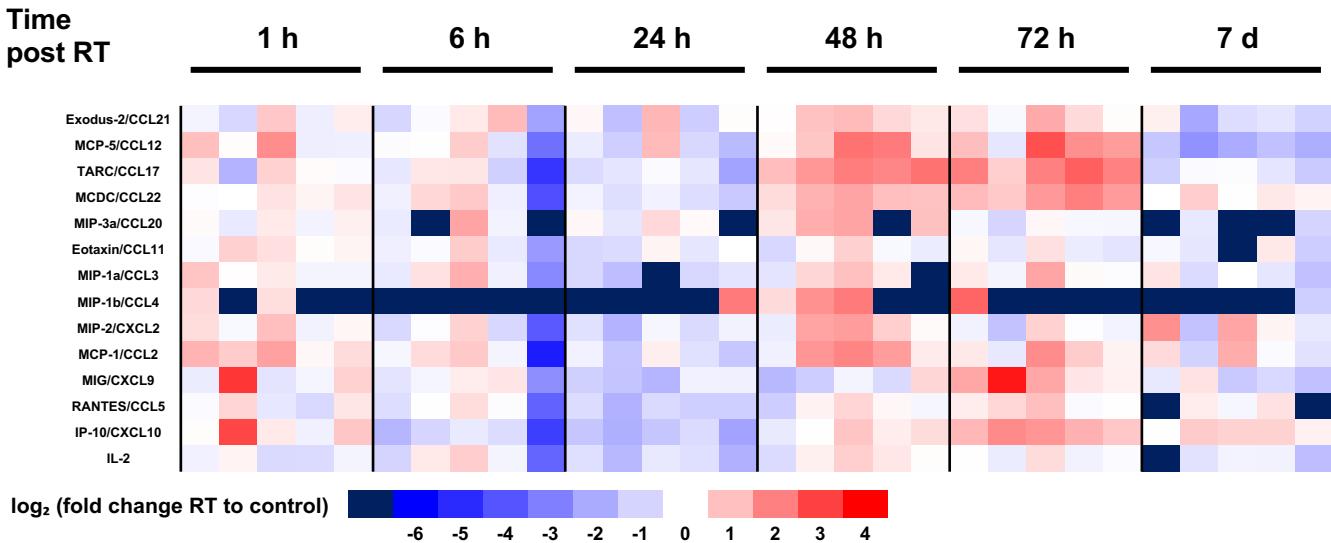
**A****B**

**Supplementary Figure 15: The effect of Fingolimod (FTY720) on peripheral blood lymphocytes counts.**

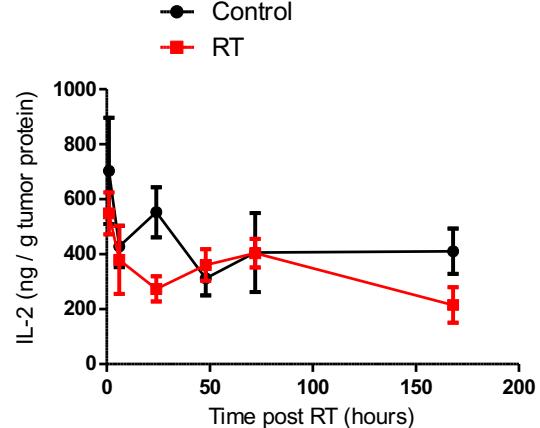
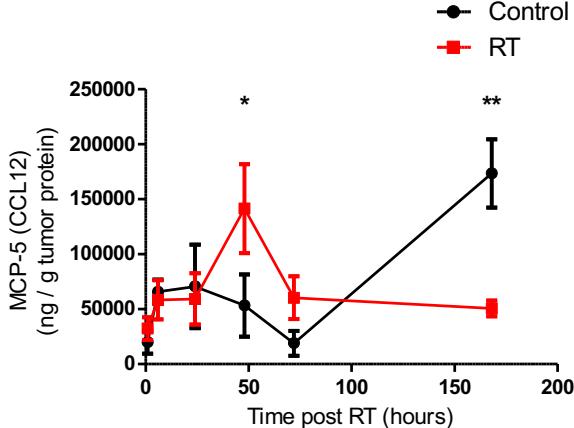
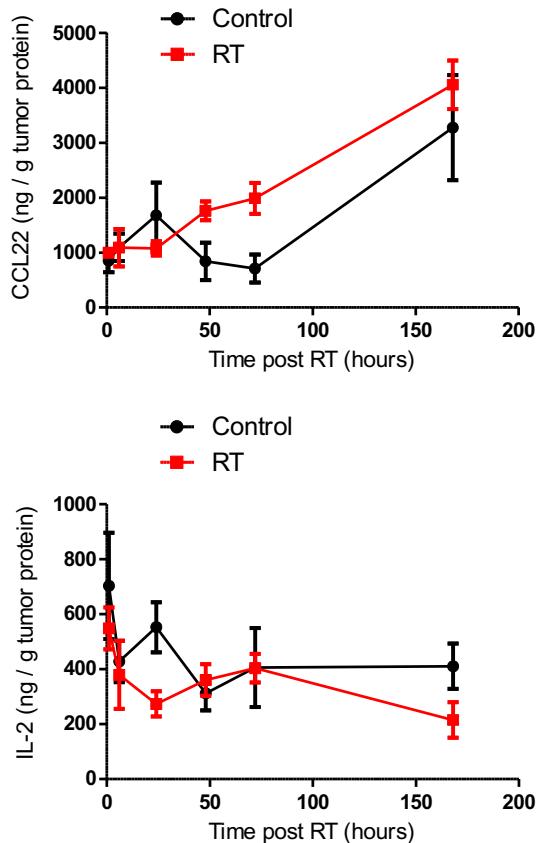
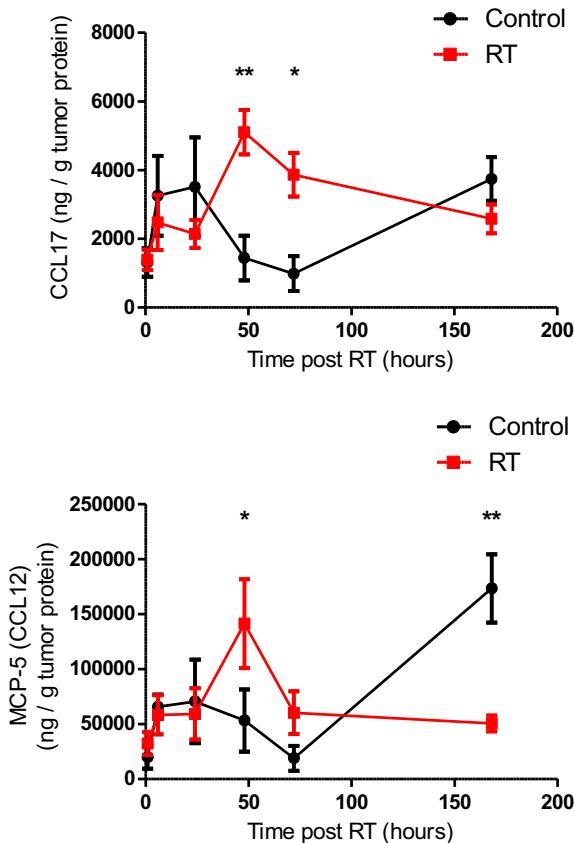
Peripheral blood samples were collected from tail veins on day 5, 7, 11, and 13. Sequestration of peripheral lymphocytes was monitored using Hemavet whole blood cell counter. Time course of the concentration of lymphocytes (A) and ratio to the baseline lymphocyte counts (B), vehicle versus FTY720-treated mice (dashed line versus solid line respectively), with or without radiation (black or red line respectively).

# Supplementary Figure 16

**A**



**B**



**Supplementary Figure 16: Chemokine/cytokine expression of a selected panel in the tumor microenvironment post-radiation.**

(A, B) Female Balb/c mice were subcutaneously implanted with  $3 \times 10^5$  RENCA cells and received 10 Gy RT as previously described. Tumor lysates were collected at different time points post RT and chemokine/cytokine (IL-2) levels were analyzed by Luminex assay. (A) Heatmap of  $\log_2$  (fold change of radiated tumor chemokine levels compared to those of non-radiated control tumors). (B) Time courses of selected chemokines/cytokines. Chemokine/cytokine level were normalized to tumor protein concentration. Non-irradiated tumors are represented by a black line, and irradiated tumors are represented by a red line, respectively. N = 4 - 5 per group. Error bars represent SEM, \*\*\*: p < 0.001, \*\*: p < 0.01, \*: p < 0.05, determined two-way ANOVA (B).